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14. ABSTRACT
This report investigated the potential role of TIRAP activation in BMF in del(5q) MDS. Using a mouse bone marrow transplant model, we have identified some key cellular mechanism in TIRAP-induced BMF. We have shown that TIRAP-induced IFNγ is a facilitator of BMF. The non-cell autonomous signaling is also responsible for the decrease in myeloid progenitor cell and the increase in progenitor apoptosis. While TIRAP-expressing myeloid cells may initiate an IFNγ response in the transplant model, non-clonal T cells are responsible for the dramatic increase in IFNγ expression. Our result also suggests that this TIRAP-induced BMF may not be through the canonical pathway. Interestingly, MDS patients with low risk for leukemic transformation also showed an enriched IFNγ signature, but not canonical innate immune signaling. We will still need to explore the signaling pathway downstream of TIRAP in our model. Finally, while there is a lack of evidence showing the presence of somatic mutations in the innate immune signaling genes in myeloid neoplasm, there is evidence of dysregulation of the pathway by aberrant epigenetic modification.

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Table of Contents

Introduction

Myelodysplastic syndromes (MDS) are among the most common of hematopoietic malignancies arisen from stem/progenitor cells. MDS may progress to either bone marrow failure (BMF) or acute myeloid leukemia (AML). However, the molecular and cellular processes involved in MDS are yet to be elucidated, and there are few effective treatment options. Our lab has identified the loss of two microRNAs miR-145 and miR-146a as contributing to the MDS phenotype by deregulation of innate immune signaling through induction of TRAF6 and TIRAP. TIRAP protein expression is higher in del(5q) patient marrow compared to controls. However, the significance of TIRAP over-expression in MDS is not known. Our objective is to investigate the mechanism of TIRAP-induced hematological abnormalities in a mouse bone marrow transplant model. We will also investigate the role of innate immune signaling in available patient data. By understanding the consequence of innate immune signal activation in MDS, a more targeted treatment plan can be developed to improve patient prognosis and quality of life.

Keywords

Myelodysplastic syndromes; Innate immune signaling; TIRAP; Interferon Gamma

Overall Project Summary

To determine the effect of toll-interleukin 1 receptor domain containing adaptor protein (TIRAP) -expression on stem/progenitor biology, to determine the role of non-cell-autonomous signal induced by TIRAP, and to determine whether TIRAP-induced bone marrow failure (BMF) is TNF receptor-associated factor 6 (TRAF6)-dependent

a) Progenitor biology of TIRAP-transplanted BMF

Mouse bone marrow cells transduced with MIG vector control or TIRAP construct were transplanted into lethally irradiated mice. At four-weeks post-transplant, we performed FACS immunophenotyping for lineage positive cells and stem/progenitor cells (LSK, $lin^-Sca\text{-}1^+c\text{-}Kit^+$), common myeloid progenitor (CMP, $lin^-Sca\text{-}1^-c\text{-}Kit^+CD34^+CD16/32^{mid}$), megakaryocyte-erythroid progenitor (MEP, $lin^-Sca\text{-}1^-c\text{-}Kit^+CD34^-CD16/32^{lo}$) and granulocyte-macrophage progenitor (GMP, $lin^-Sca\text{-}1^-c\text{-}Kit^+CD34^+CD16/32^{hi}$). The TIRAP-expressing cells (GFP+) have showed marked decreased in erythroid cells and mature granulocytes. The TIRAP-transduced cells also affected the co-transplanted GFP- cells, resulting in decrease in myeloid, erythroid, megakaryocytic and B cells, with increase in T cells. The increase of GFP- T cells could come from an increased expansion and activation of resident T cells in the bone marrow; or alternatively it can represent a recruitment of recipient-derived T cells. Notably, several studies has demonstrated increased T cells with cytotoxic activity in low risk MDS patient (summarized in Aggrewal, 2011). It is possible that increased T cell population can be a cause for the hypocellularity observed in TIRAP-transplanted mice. The identity and functional relevance of the GFP- T cell population will be topic of further investigation in this lab.

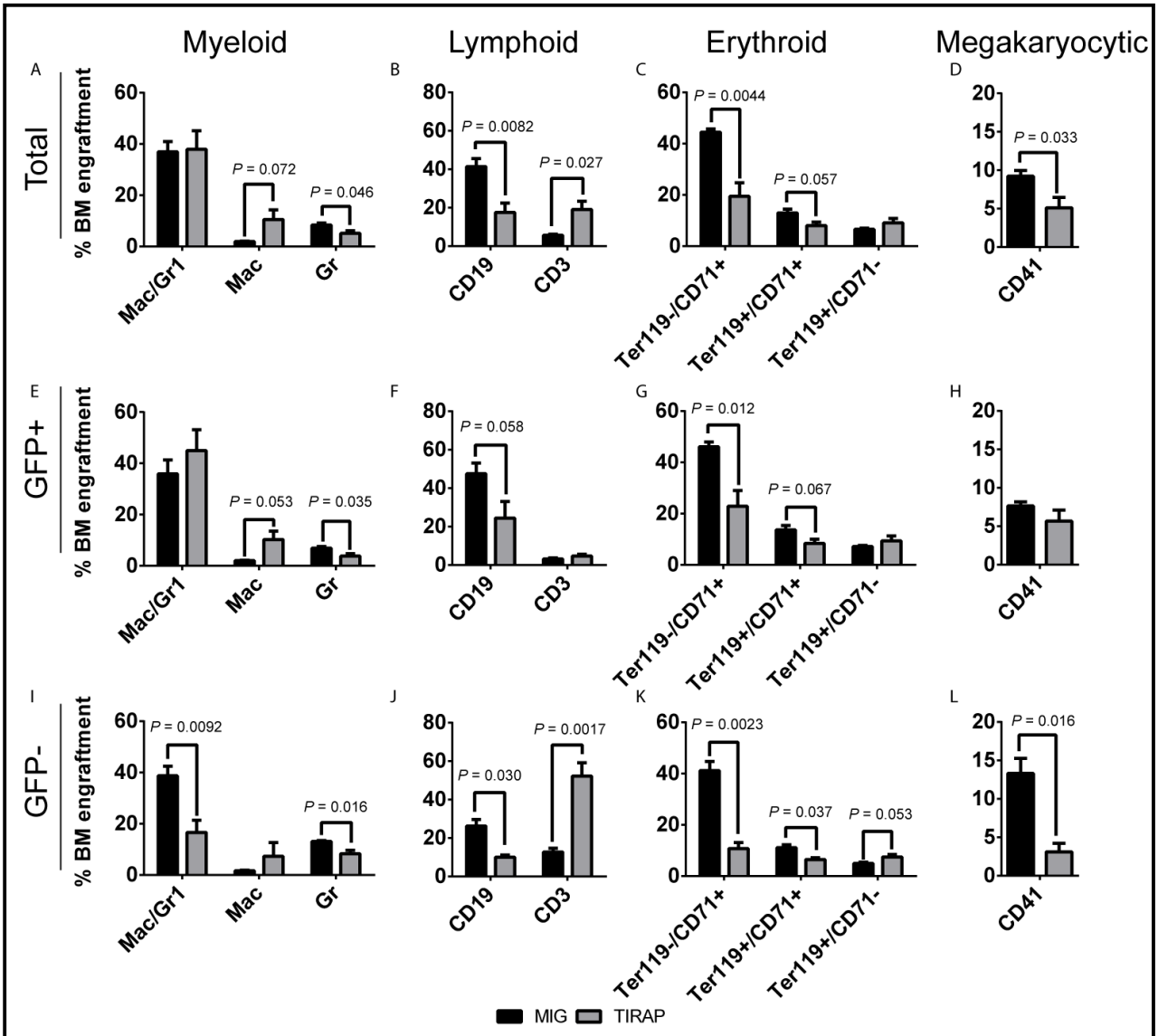

Figure 1. Immunophenotyping of mice transplanted with TIRAP-transduced bone marrow cells. The percentage of viable (DAPI-) mononuclear bone marrow cells with each lineage markers were determined. The GFP+ and GFP- population were analyzed separately.

TIRAP-transplanted mice had less myeloid progenitor cells overall. There is a decrease of all progenitor cell types in the GFP+ (therefore donor-derived) population, especially in the moribund animals (Figure 2). Interestingly, there is also a significant decrease of GMP in the GFP- (recipient-derived) cells. This observation agrees with the cell non-autonomous effect we have observed in the mature cells in the TIRAP transplant model.

The bone marrow was also stain for activated caspase 3 and Ki67 for apoptosis and proliferation. There is an increase in apoptosis in all myeloid lineage progenitor cells regardless whether the cells are of donor or recipient origin (Figure 3). This suggests that the decrease in progenitor cell count and the bone marrow failure phenotype is brought on, at least in part, by cell death in both normal and TIRAP-expressing progenitor cells.

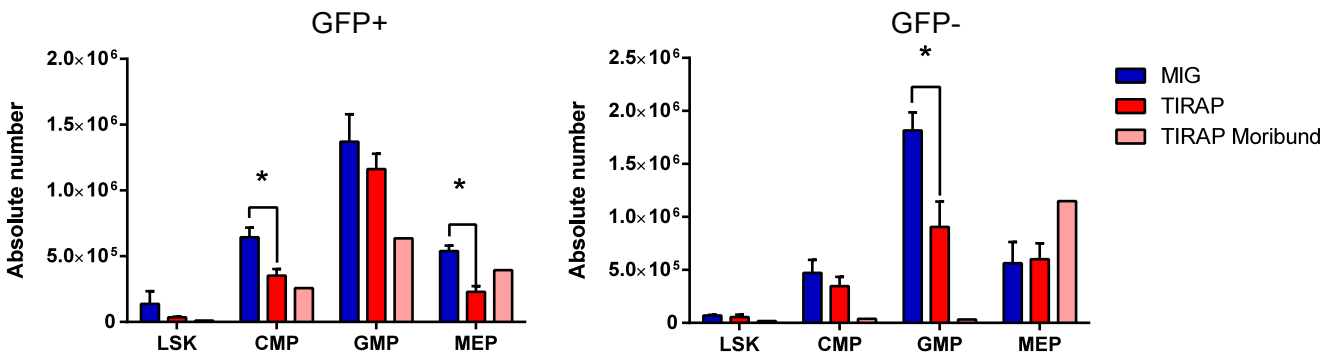

Figure 2. Numeration of progenitor populations 4 weeks post-transplant. The percentage of viable (DAPI-) mononuclear bone marrow cells with each progenitor immunophenotypic markers were determined. The GFP+ and GFP- population were analyzed separately. The absolute number of progenitor cells in each sample were determined by multiplying viable cell count by the percentage of each progenitor population.

A published study by Pang et al. showed that in low-risk MDS patients, there is a marked decrease of bone marrow GMP compared to normal age-matched bone marrow (Pang, 2013). The same study also identified an increase in apoptotic CMP, GMP and MEP in low-risk MDS bone marrow mononuclear cells. However, analysis is not performed in the CD34+CD38- population, which is closest resembling the mouse LSK population. This suggests that the TIRAP transplanted mice bone marrow progenitor cells can phenocopy the apoptotic phenotype of low-risk MDS patients.

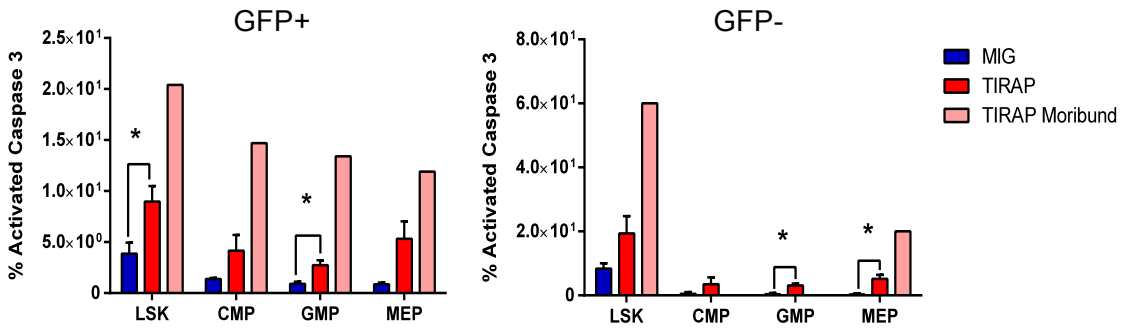

Figure 3. Apoptosis of progenitor populations 4 weeks post-transplant. The percentage of mononuclear bone marrow cells with activated caspase 3 staining within each progenitor population was determined. The GFP+ and GFP- population were analyzed separately.

We attempted to investigate the proliferation status of the bone marrow myeloid progenitor cells after transplant using Ki67 staining. However, at 4-weeks post-transplant, the percentage of proliferating progenitor cells are very high, representing the period before the bone marrow is stabilized after transplantation. There is a trend of lower proliferative potential in the recipient-derived LSK population in TIRAP-transplanted mice. However, further examination is required. We are in the process of optimizing the dosage of timing of BrdU incorporation assay for a more accurate measurement of actively dividing cells.

Group	Dose	Positive	Stem Cell Frequencey	P-value
MIG	150	22/22	1/16.8	0.162
	75	21/22		
	37.5	19/22		
	18.75	8/10		
	9	7/10		
TIRAP	150	22/22	1/11.7	
	75	22/22		
	37.5	19/22		
	18.75	10/10		
	9	8/10		

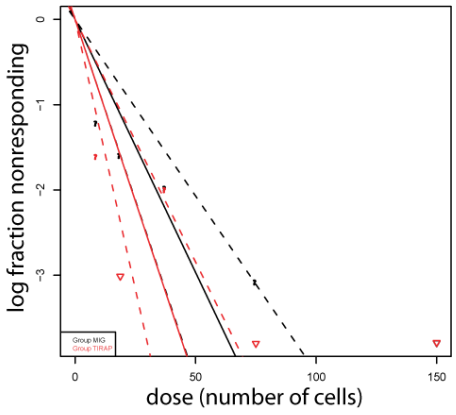

Figure 4. LT-CIC assay on TIRAP-transduced bone marrow cells. MIG or TIRAP-transduced 5FU-bone marrow cells were cultured on irradiated feeder cells for 4 weeks. Colony-forming units were determined by differentiating in MethoCult® media.

We also performed LT-CIC assay to examine the activity of hematopoietic stem progenitor cells (HSPC) in TIRAP-transduced bone marrow (Figure 4). 5FU-injected bone marrow showed high proportion of colony-forming cells after long-term culture in both MIG and TIRAP-transduced cells. This result suggests that TIRAP-transduced bone marrow cells do not intrinsically have

lower HSPC number and activity. The decrease of number and activity shown in transplanted mice is most likely the result of extrinsic factors from the bone marrow niche in TIRAP-transplanted mice. The investigation of the niche effect is outside of the scope of present project and is being followed up by other investigators in this lab.

b) Characterization of the TIRAP-induced cell non-autonomous signaling We have previously shown that TIRAP expression induces expression of cytokines IFNγ and IL10 in mouse bone marrow cells. We attempted to identify the cell type that is responsible for the cytokine secretion. Mouse bone marrow cells were transduced with MIG control and TIRAP and cultured. The cells were then FACS sorted based on Mac/Gr-1 marker (for myeloid cells, GM+) and CD3 marker (for T cells). Total RNA was extracted from sorted cells and quantitative RT-PCR was performed for IFNγ expression.

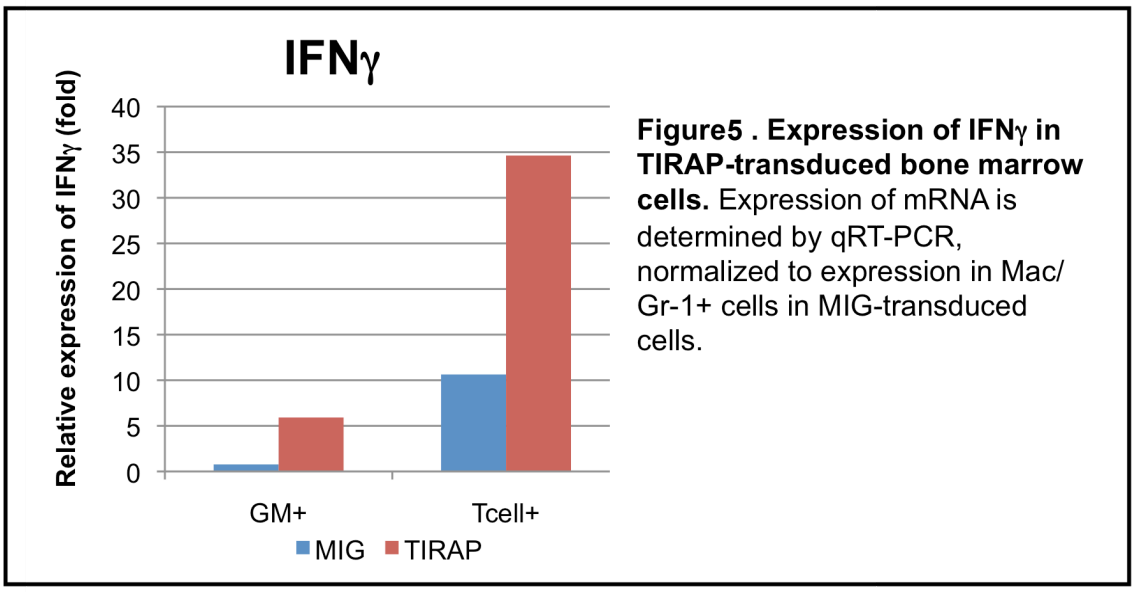

Figure5 . Expression of IFNγ in TIRAP-transduced bone marrow cells. Expression of mRNA is determined by qRT-PCR, normalized to expression in Mac/ Gr-1+ cells in MIG-transduced cells.

In one preliminary experiment, both TIRAP-expressing myeloid and T cells have more IFNγ transcripts compared to MIG control transduced cells (Figure 5). However, the level of IFNγ transcript is relatively higher in the CD3+ T cell population, suggesting that TIRAP expressing T-cells are the major source of IFNγ in the transduced bone marrow cells. However, our study looking at HSPC activity also suggests that TIRAP-expressing cells could have an effect on the bone marrow niche in the transplant model. To examine the full effect of TIRAP-induced IFNγ in the mouse transplant system, we would need to assay for IFNγ expression *in vivo*. In addition, the *ex vivo* experiment can only detect IFNγ transcript level. In order to determine the protein level of different cell type in the TIRAP-transplanted mice, we performed intracellular IFNγ staining by flow cytometry, hoping to combine cell markers and IFNγ protein detection in 4-week post-transplant mice. However, due to the low amount of endogenous cytokine expression and the level of detection that can be achieved by intracellular staining followed by flow cytometery, we are not able to conclusively detect IFNγ in transplanted bone marrow.

Alternatively, we have isolated various cell populations from transplanted mouse bone marrow (T cells, B cells, monocytes, granulocytes, and nature killer (NK) cells) by FACS to examine cytokine expressions (Figure 6). Again, we observed an increase in GFP- T cell number in the TIRAP-transplanted mice. In addition, we observed a decrease in NK cell (CD49b+), which is

another cell type that frequently expresses IFNγ, in the bone marrow of TIRAP-transplanted mice (Figure 6). Absolute quantification of IFNγ transcript level showed increased in IFNγ expression from both TIRAP-expressing myeloid and T cells compared to MIG-expressing counterparts, where IFNγ expression is only detectable in the T cell population. Interestingly, the GFP- population in the TIRAP-transplanted mice also showed increase in IFNγ expression in myeloid and T cell, suggesting that IFNγ may be involved in TIRAP-induced cell non-autonomous phenotypes. Overall, the GFP- T cell population is responsible for the majority of the IFNγ transcript detected in TIRAP-transplanted mice, while myeloid cells is the major producer of IFNγ in TIRAP-expressing cells. Previous study on MDS patient bone marrow showed that the majority of IFNγ-producing cells are CD68+ macrophage cells (Kitagawa, 1997).

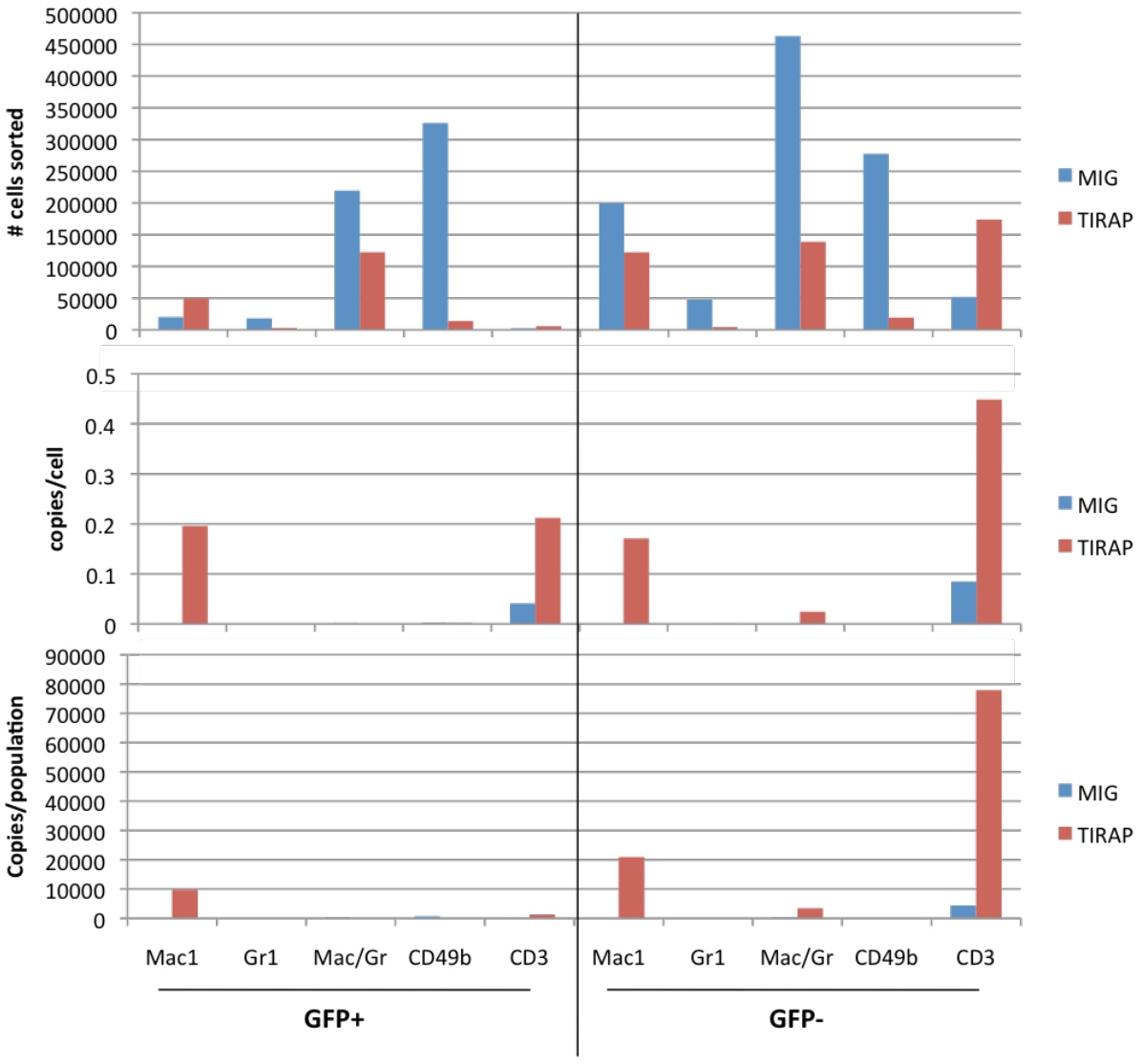

Figure 6. Quantification of IFNγ expression in mice transplanted with TIRAP-expressing bone marrow. Bone marrow from 4-week-tranplanted mice were flow sorted and total RNA were isolated. qRT-PCR for IFNγ were performed against known copy number standards.

c) Examination of the TIRAP-TRAF6 signaling axis in TIRAP-induced BMF We have previously observed that, unlike TRAF6-induced signals, TIRAP expression did not induce expression of IL-6. IL-6 secretion was found to be important in the dysplastic phenotype and BMF in TRAF6-transplanted mice (Starczynowski, 2010). We transduced mouse bone marrow cells with TIRAP-expressing retrovirus and then assay for ubiquitination of TRAF6 as an indication of TRAF6 protein activation. In TIRAP-expressing cells, we observed increased amount of ubiquinated TRAF6 (data not shown), showing that TIRAP expression in mouse bone marrow is able to activate TRAF6 and presumably signaling downstream of TRAF6. We transduced mouse bone marrow cells with TIRAP and a dominant-negative construct of TRAF6 (TRAF6-DN) to determine whether the cytokines induced by TIRAP expression is through downstream TRAF6 signaling.

Interestingly, TIRAP-induced expression of IL-10 and IFNγ were found to be independent of TRAF6 activity (Figure 7). While TRAF6-DN alone does not modulate expression level of either cytokines, blocking TRAF6 in TIRAP-expressing cells enhanced IFNγ induction. Another study has shown that in TRAF6-deficient Th1 cells IFNγ production by IL-18 stimulation was dampened, suggesting that in TIRAP-expressing mouse bone marrow cells, TRAF6 acts to block IFNγ in a context dependent fashion (Chiffoleau, 2003). Moreover, by using two independent MyD88 dominant-negative constructs (MyD88N and MyD88C), we showed that blocking of MyD88-dependent signaling also enhanced the production of both cytokines (Figure 7). All together, our data suggests that TIRAP induces IFNγ and IL-10 independent of the canonical MyD88-TRAF6 dependent signaling.

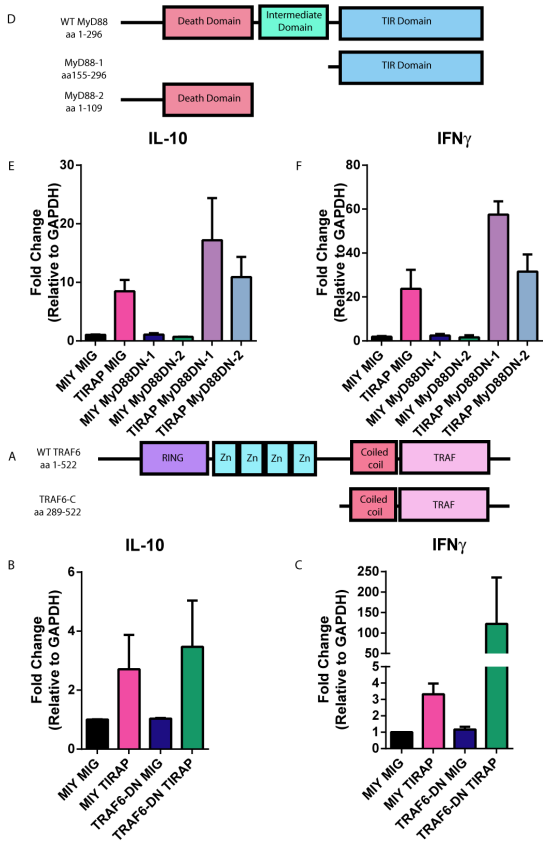

Figure 7. TIRAP induces IL10 and IFNγ independent of TRAF6 or MyD88 activity. Mouse bone marrow cells transduced with TIRAP or MIG control were co-transduced with control or TRAF6-DN (B,C) or with control or MyD88-DN constructs (E,F). qRT-PCR was performed to determine relative expression of IL10 and IFNγ transcripts.

To follow-up on this line of investigation, we will investigate the TIRAP-induced downstream signaling by luciferase assay. We have obtained a lentivirus based luciferase vector to more effectively introduce promoter-luciferase construct into mouse bone marrow cells. We are in the process of cloning NFkB, IRES, AP-1 and CREB promoter into a lentiviral luciferase vector. There are also challenges in producing lentivirus of sufficient quantity and quality to transduce mouse bone marrow cells. We are currently working on optimizing this procedure.

<u>To characterize TIRAP-induced myeloproliferation in IFNγ-KO mice, to determine the transplantability of the myeloprolifation, and to determine the downstream signaling activated by TIRAP in myeloproliferation</u>

a) Further characterization of TIRAP-induced myeloproliferation We have previously shown that TIRAP-expression in IFNγ knock-out mouse bone marrow transplant does not lead to BMF phenotype, while IL-10 knock-out TIRAP transplants showed similar results as transplants with wildtype donor bone marrow expressing TIRAP in our preliminary study.

We have performed more biological replicates of the transplants and followed the transplanted mice further, which enable us to observe the full spectrum of phenotypes observed in transplanted mice with IL-10 or IFNγ knockout mice as donors.

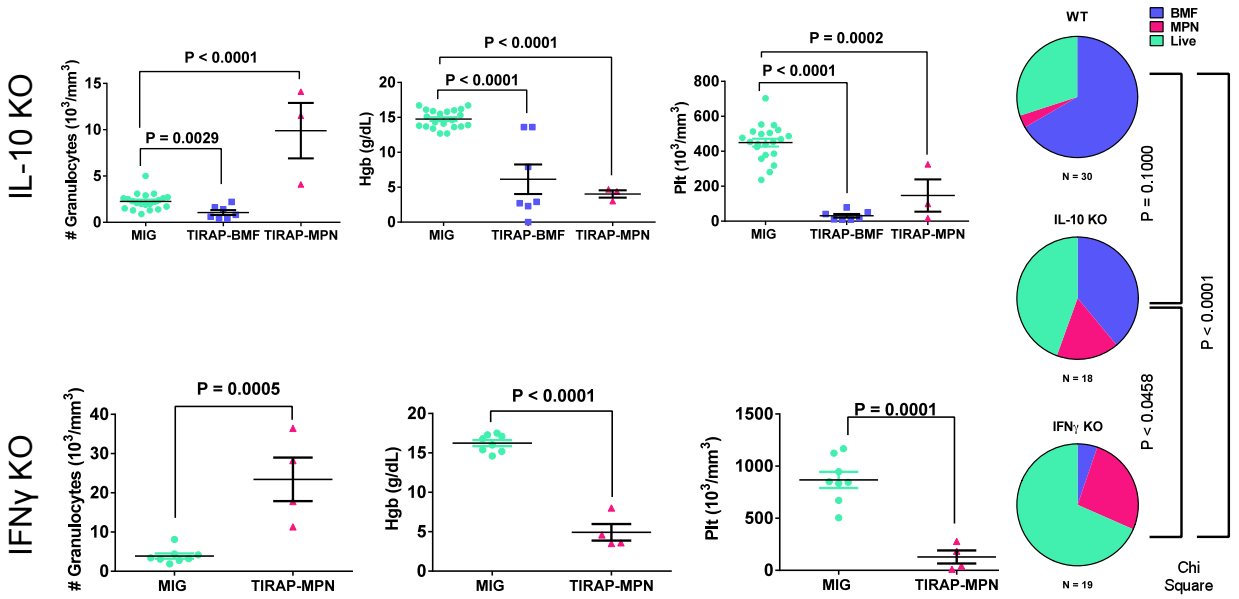

Figure 8. TIRAP-induced BMF phenotype is dependent on IFNγ expression. Complete blood count of mice post-transplant was used to monitor for pancytopenia or leukocytosis. The proportions of mice developing BMP or myeloproliferation from transplant of TIRAP-expressing wildtype, IL10 knockout and IFNγ knockout mouse bone marrow cells were compared by Chi-square test.

Mice transplanted with TIRAP-expressing IFNγ-knockout mouse bone marrow rarely develop BMF (Figure 8), which is significantly different from transplants with wildtype or IL-10 knockout donor cells. Although not statistically significant, mice transplanted with IL-10 knockout TIRAP-

expressing bone marrow may also have an elevated probability to develop myeloproliferation. This observation may be linked to the classical role of IL-10 as a myelo-suppressive cytokine.

We are currently exploring the myeloproliferative phenotype with flow cytometry-based progenitor staining, apoptosis and proliferation analysis as described previously. However, we have had problem with the mouse colony maintenance and is currently refreshing our knockout mouse colonies with new breeders from JAX. This will also curtail any effects from possible genetic drift between our in-house colonies and the original JAX strain.

b) Clinical correlation of cytokine dysregulation in MDS and myeloproliferation Our study in the mouse bone marrow transplant system suggests an important role for IFNγ in TIRAP-induced BMF. Interestingly, in MDS patients with low risk for leukemic transformation (including -5q patients), there is a strong correlation between TIRAP and IFNG expression (Figure 9). There is also enrichment for IFNγ-stimulated signature in the low risk MDS patient sample compared to healthy controls (Figure 9). However, no enrichment was found for IL10 pathway or the canonical innate immune signal carried by NFκB pathway. Furthermore, the IFNγ signature is also enriched in MDS patient samples compared to AML patient samples, suggesting the loss of IFNγ signal leads to a more aggressive proliferative phenotype (data not shown). The role of IFNγ in leukemic transformation of MDS could be a topic of further investigation.

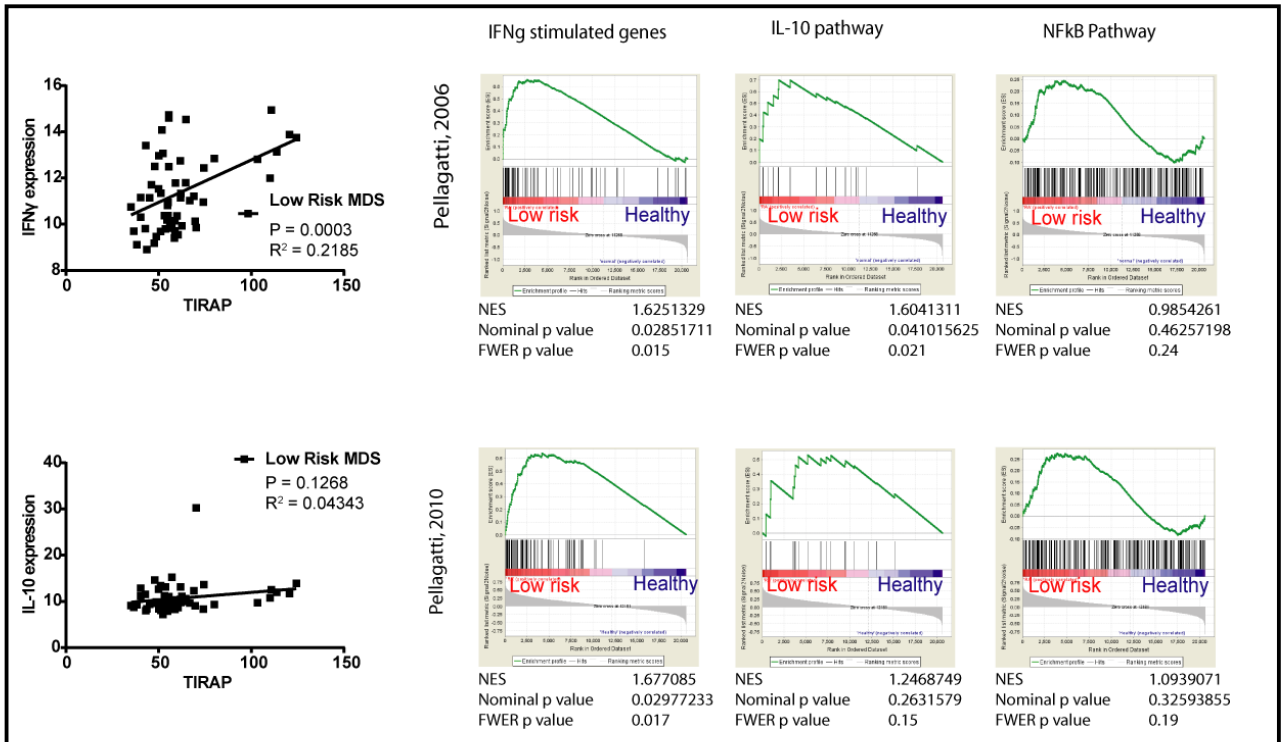

Figure 9. Gene Set Enrichment Analysis (GSEA) showed enrichment of IFNg signature in low risk MDS patients compared to healthy controls. Microarray data from two studies from the Boultwood lab were used as input for the analysis (Pellagatti, 2006, Pellagatti, 2010).

Identification additional innate immune signal activation mutation in MDS patients

a) Identification of mutations in the innate immune pathway genes in myeloid leukemia in TCGA database Using available public database of somatic mutations found in acute myeloid

leukemia patients, we only detected one case of frameshift mutation in TLR4 that would suggest an loss-of-function mutation in 200 AML patients. This finding showed that somatic mutation of innate immune pathway genes is not frequent or prominent in AML. We are in the process of examining other publically available sequencing data on MDS or myeloproliferative neoplasm patients for innate immune signaling gene mutations.

A publication by Garcia-Manero group from MD Anderson showed mutations discovered in TLRs in MDS and an elevated level of TLR2 and partners TLR1 and TLR6 in MDS patients. High level of TLR2 expression is correlative to low-risk MDS, which is more likely to develop BMF compared to progression to AML (Wei, 2013). Although this study did not examine the level of TIRAP in MDS, TLR2 is one of the receptors that signals through TIRAP, resulting in downstream signal activity. This study partially confirms with our observation of the importance of innate immune activity in MDS.

b) Analysis of promoter methylation data from study by ME Figueroa (MDS and AML) and TCGA (AML with mutation data) Recent genomic studies in myeloid neoplasm has brought to light the importance of aberrant promoter methylation in disease initiation. Thus we begin to explore the possibility that innate immune signaling is being modulated by aberrant epigenetic modification.

Analysis of promoter methylation status using previously published data showed that TLR2 is hypermethylated compared to normal CD34 cells in AML samples (Figure 10), but not MDS samples (Figueroa, 2010). The hypermethylation of the promoter will result in a lower transcriptional level. This suggests that signaling through TLR2 is downregulated in AML cells compared to normal hematopoietic progenitors.

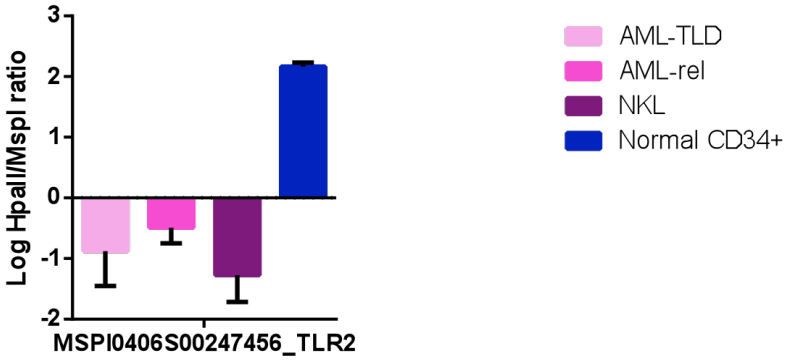

Figure10 TLR2 promoter is hypermethylated in AML blasts compared to normal CD34+ cells. DNA methylation status was determined by methylation-sensitive restriction enzyme analysis followed by genomic DNA array.

Using DNA methylation data from the TCGA dataset for AML, we observed correlation, even within the AML patient group, between recurrent mutations in PHF6 and IDH2 with hypermethylation of TLR2 promoter region (Figure 11). Mutation in PHF6 has been associated

to lower overall survival (Patel, 2012). Also, expression of TLR2 in the AML TCGA dataset is negatively correlative to the mutational status of TP53, which is another poor prognostic marker. All together, these analyses with available AML data suggests that signaling through TLR2 is down regulated in AML and that lower TLR2 level is often associated with poor prognostic markers. Using the publically available databases, we can explore further potential effects of epigenetic modification of innate immune signaling pathways.

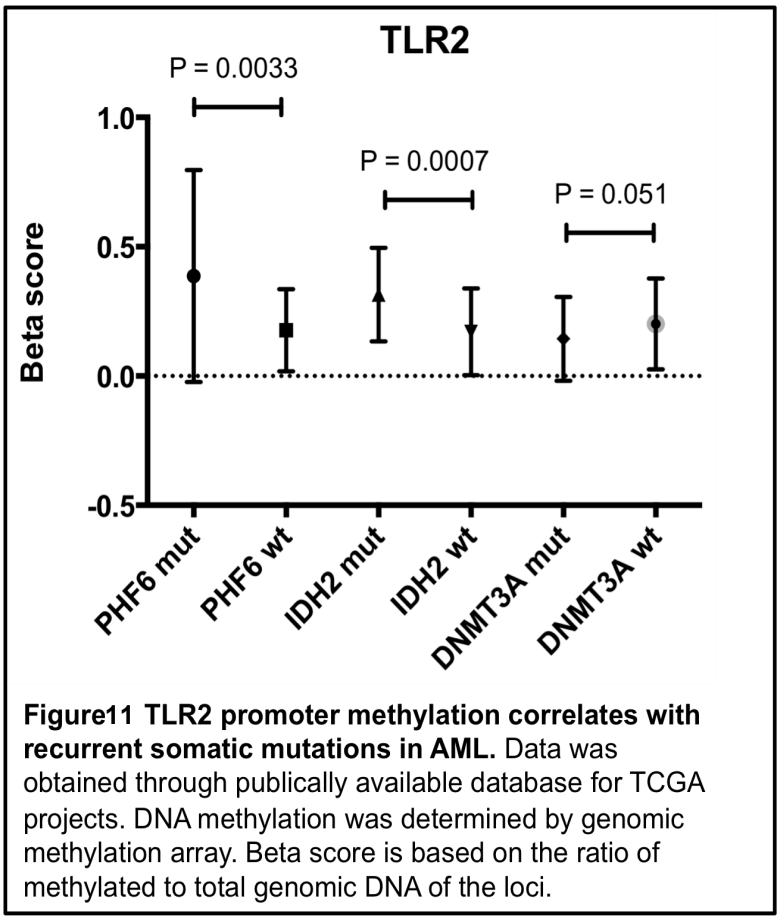

Figure11 TLR2 promoter methylation correlates with recurrent somatic mutations in AML. Data was obtained through publically available database for TCGA projects. DNA methylation was determined by genomic methylation array. Beta score is based on the ratio of methylated to total genomic DNA of the loci.

Key Research Accomplishments

- IFNγ is a key molecule in TIRAP-induced bone marrow failure
- TIRAP-induced bone marrow failure is brought on by apoptosis of myeloid progenitor cells
- TIRAP-induced phenotype is independent of downstream TRAF6 signaling
- IFNg response
- Innate immune signaling molecules are dysregulated in MDS and AML, partially through modulation of promoter methylation

Conclusion

This report investigated the potential role of TIRAP activation in BMF in del(5q) MDS. Using a mouse bone marrow transplant model, we have identified some key cellular mechanism in

TIRAP-induced BMF. We have shown that TIRAP-induced IFNγ is a facilitator of BMF. The non-cell autonomous signaling is also responsible for the decrease in myeloid progenitor cell and the increase in progenitor apoptosis. While TIRAP-expressing myeloid cells may initiate an IFNγ response in the transplant model, non-clonal T cells are responsible for the dramatic increase in IFNγ expression. Our result also suggests that this TIRAP-induced BMF may not be through the canonical pathway. Interestingly, MDS patients with low risk for leukemic transformation also showed an enriched IFNγ signature, but not canonical innate immune signaling. We will still need to explore the signaling pathway downstream of TIRAP in our model. Finally, while there is a lack of evidence showing the presence of somatic mutations in the innate immune signaling genes in myeloid neoplasm, there is evidence of dysregulation of the pathway by aberrant epigenetic modification.

Publications, Abstracts, and Presentations

Not applicable, although manuscript is in preparation.

Inventions, Patents and Licenses

Not applicable

Reportable Outcomes

Not applicable

Other Achievements

Not applicable

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Appendices

Not applicable.